Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance

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Summary

While tumors become addicted to oncogenes like *Ras*, the microenvironment in which tumor cells reside changes during tumorigenesis; the cells are surrounded initially by normal tissue and later by tumor tissue. Hence, we asked if Ras exerts its oncogenic effects through the same set of effectors during different stages of tumorigenesis. We now show in human cells that the Ras effector pathways MAPK, RalGEF, and PI3K are required to initiate tumor growth. Conversely, activation of the PI3K/AKT pathway replaced Ras once tumors formed, although other effectors were still activated independently of Ras, presumably by factors provided upon the establishment of a tumor microenvironment. Thus, as tumorigenesis progresses the addiction of cancers to their initiating oncogene is reduced to, at least in the case of *Ras*, the PI3K/AKT pathway.

Introduction

The small GTPase Ras is commonly mutated in human cancers, often glycine[12] to valine (G12V), to remain in the constitutively active oncogenic GTP bound state (Bos, 1989). It has long been appreciated that expression of this active version of Ras promotes tumor initiation by activating at least three different effectors based on the work from many labs using Ras effector binding mutants, dominant-negative and constitutively active proteins downstream of Ras, and pharmacological inhibitors: Raf, PI3-kinase (PI3K), and RalGEFs (Shields et al., 2000; Ulku and Der, 2003). Raf is a serine/threonine kinase that is localized to the plasma membrane from the cytoplasm and activated by GTP-Ras. Activated Raf proteins then initiate a MAP kinase (MAPK) signal transduction cascade that leads to transformed morphologies, anchorage-independent growth, and angiogenesis (Morrison and Cutler, 1997; Shields et al., 2000). The PI3K is also activated via interaction of the p85 regulatory subunit with GTP-Ras, leading to the phosphorylation of phosphoinositides, creating multiple cascades that lead to changes in cell morphology, as well as fostering angiogenesis and cell survival (Luo et al., 2003). Lastly, RalGEFs are a family of five guanine exchange factors (GEFs) that are activated via their recruitment to the plasma membrane by GTP-Ras, where they activate RalA and RalB, the former of which appears to be required in tumorigenesis (Feig, 2003; Lim et al., 2005).

It is well established that Ras promotes the *initiation* of tumorigenesis. However, patients are diagnosed and treated once tumors have already been established, during what is termed tumor *maintenance*. In this regard, mice harboring a doxycycline (dox)-inducible oncogenic version of *Ras* grew tumors (in the absence of the tumor suppressor INK4a) upon ingesting dox, but when these treatments were ceased the tumors regressed (Chin et al., 1999). It was concluded that tumors become "addicted" to oncogenic Ras, requiring this protein to both initiate and maintain tumor growth (Chin et al., 1999), as is the case for other oncogenes (Giuriato et al., 2004).

Despite the fact that oncogenic *Ras* expression is required during tumor initiation and maintenance, the normal microenvironment for which a tumor initiates is vastly different from that in which a tumor is maintained. In particular, the altered microenvironment of the tumor can play a positive role in tumor growth. The stroma of a tumor is aberrant, having appearances of undergoing a wound healing response (Dvorak, 1986) in which cell proliferation can be stimulated and tumor growth enhanced (Mueller and Fusenig, 2004). Mast cells are recruited to the tumor and then release metalloproteinases, which in turn free growth and angiogenic factors trapped in the ECM to promote tumor growth and progression (Coussens and Werb, 2002). The vasculature can supply a host of survival- and growth-promoting factors (Bergers and Benjamin, 2003). Even

SIGNIFICANCE

Tumorigenesis is a dynamic process, and we show that this translates at the molecular level to differential requirements of *Ras* oncogene signaling during various stages of tumor growth. Since cancers are treated once they have been established, and Ras signaling was reduced to activation of PI3K/AKT in established tumors, the PI3K/AKT pathway may be particularly important for the treatment of *Ras*-driven human cancers. The reduction of oncogene addiction during the dynamic growth of the solid tumors appeared to be due to activation of Ras effectors by the tumor environment. This environment even fostered the tumorigenic proliferation of otherwise nonmalignant cells in what can be best described as parasitic growth, which may reflect the known heterogeneity in the neoplastic fraction of human tumors.

the hypoxic environment of tumors can activate the transcription factor HIF-1α, and correspondingly a series of genes to counter hypoxia that are advantageous to the tumor, including those that foster angiogenesis, cell proliferation and survival, and glycolysis, to name a few (Harris, 2002).

Given that the microenvironment in which tumor cells reside changes as a tumor becomes established, we asked whether Ras exerts its oncogenic effects through the same set of effectors during tumor initiation and maintenance. However, any one of the three major Ras effectors alone can promote tumor initiation and hence presumably tumor maintenance of transformed murine cells (Rangarajan et al., 2004) and, although with different kinetics, also in rat mammary glands in vivo (McFarlin and Gould, 2003; McFarlin et al., 2003). Indeed, transformation and tumorigenesis are typically more lax in rodent cells than in human cells (Lim and Counter, 2004; Rangarajan and Weinberg, 2003). We therefore chose to focus our studies on human cells. In this regard, expression of the SV40 viral oncoproteins T- and t-Ag and the mammalian protein hTERT (the catalytic subunit of telomerase) in conjunction with oncogenic (G12V) Ras (Ras^{G12V}) drives normal human cells from a wide spectrum of cell types to a tumorigenic state (Hahn et al., 1999; O'Hayer and Counter, 2005), providing a simplified and defined system to explore Ras oncogenesis in human cells (Hamad et al., 2002). Using this system, we replaced Ras with different combinations of molecules that activate Ras effector pathways to determine which signals were required to initiate tumor growth and, by generating tumors with an inducible Ras protein, which pathways maintained tumor growth once Ras expression was silenced.

Results and discussion

Multiple Ras effectors are required to initiate tumor growth of human cells in vivo

To explore the requirements of oncogenic Ras signaling during the dynamic process of tumorigenesis, we sought to identify the signals activated by oncogenic Ras that are required to initiate versus maintain tumor growth of human cells. To first identify the pathways required for tumor initiation, we replaced oncogenic Ras with molecules that preferentially activate PI3K, MAPK, or RalGEF pathways in primary human embryonic kidney cells engineered to express T-Ag, t-Ag, and hTERT (termed TtH cells). Since tumor growth of TtH cells was already shown to minimally depend upon expression of the E37G mutant of oncogenic Ras (Hamad et al., 2002), which primarily activates RalGEFs (White et al., 1996), we tested whether activation of the PI3K pathway, the MAPK pathway, or both cooperate with $Ras^{G12V,E37G}$ to initiate tumor growth. $Ras^{G12V,E37G}$ was stably coexpressed in TtH cells with either a control vector, an activated form of Raf-1 (ΔRaf22W) to stimulate the MAPK pathway (Stanton et al., 1989), an activated form of PI3K (p110-CAAX) to stimulate the PI3K pathway (Rodriguez-Viciana et al., 1997), or both ΔRaf22W and p110-CAAX. As further controls, TtH cells harboring vector alone or a vector encoding oncogenic Ras were generated. The resultant six polyclonal cell lines were confirmed to express the desired transgenes (Figure 1A) and assayed for activation of the MAPK and PI3K pathways by measuring the levels of phosphorylated ERK1 and 2 (ERK1/2) and phosphorylated AKT, respectively. As expected, ERK1 and ERK2 were specifically activated only in cells expressing ΔRaf22W, whereas AKT was weakly activated in all cells, apparently due to the leaky nature of the $Ras^{G12V,E37G}$ mutant, although AKT was clearly the most phosphorylated in cells expressing p110-CAAX (Figure 1B).

The six cell lines were each injected subcutaneously into four immunocompromised mice, and tumor growth was monitored over time. Only cells in which all three Ras effector pathways were activated were tumorigenic. Specifically, cells coexpressing $Ras^{G12V,E37G}$, ΔRaf22W, and p110-CAAX were the most aggressive followed by cells coexpressing $Ras^{G12V,E37G}$ and ΔRaf22W (Figure 1D), in which PI3K was weakly activated by the $Ras^{G12V,E37G}$ protein (Figure 1B). PI3K activation was shown to be required in the latter cells, as diminishing AKT activation by expression of PTEN, a negative regulator of this pathway (Vivanco and Sawyers, 2002), abolished the tumorigenic growth of these cells (Figures 1C and 1D). Since robust activation of any two of the RalGEF, PI3K, or MAPK pathways failed to initiate tumor growth (Figure 1D and Hamad et al., 2002), we conclude that these three effectors are minimally required to initiate human tumor cell growth of TtH cells. In agreement, multiple Ras effectors have been reported to drive a variety of human cells expressing hTERT, T-Ag, and t-Ag to a tumorigenic state (Rangarajan et al., 2004).

Oncogene addiction in human tumor cells

To determine if Ras was required during tumor maintenance in human cells, a 4-hydroxytamoxifen (4-OHT)-inducible ER:Ras^{G12V} fusion protein (Dajee et al., 2002) comprised of a mutant form of the estrogen receptor ligand binding domain and oncogenic Ras was expressed in TtH cells. As a negative control, TtH cells were stably infected with a control retrovirus, and as a positive control, the cells were stably infected with a retrovirus encoding Ras^{G12V}. Like vector control cells, in the absence of the 4-OHT the fusion protein was undetectable and did not stimulate the MAPK, PI3K, or RalGEF pathways, as measured by an increase in AKT and ERK1/2 phosphorylation and the level of GTP bound RalA, respectively (Figures 2A and 3A). This inability to activate Ras effectors was reflected in assays of Ras oncogenesis. In the absence of 4-OHT, ER:Ras^{G12V}-TtH cells behaved like vector control cells and failed to grow in soft agar (Figure 2B and data not shown) or form tumors in mice (Figure 2C). Conversely, binding of 4-OHT by the ER portion of the fusion protein relieved this inhibition, resulting in detectable ER:Ras^{G12V} protein that behaved like positive control Ras^{G12V}, activating Ras effectors (Figures 2A and 3A) and promoting anchorage-independent (Figure 2B and data not shown) and tumorigenic growth (Figure 2C). We next addressed whether Ras was required for tumor maintenance, and here we show that inducible inhibition of ER:Ras^{G12V} *after* tumors had formed by halting 4-OHT treatments led to tumor regression (Figure 2C). Thus, as in murine models (Chin et al., 1999; Tarutani et al., 2003), human cells become addicted to oncogenic Ras for maintenance of tumorigenic growth.

Activation of the PI3K pathway maintains tumor growth in the absence of oncogenic Ras

We next tested which Ras effector pathway(s) need to be activated to overcome the tumor regression observed upon suppressing ER:Ras^{G12V} expression in established tumors. Two independent approaches were used to preferentially activate one arm of Ras oncogenic signaling in ER:Ras^{G12V} TtH cells: acti-

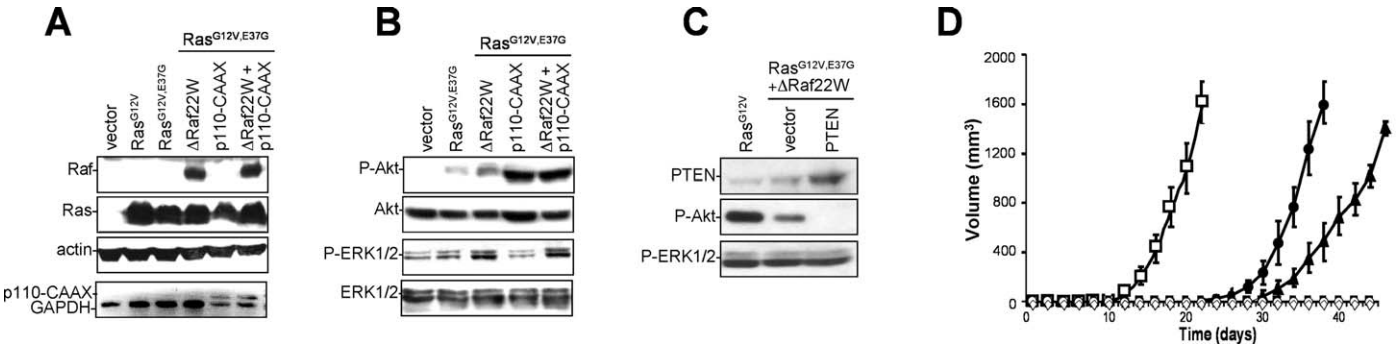

Figure 1. Tumor initiation is mediated by multiple Ras effectors

A: Detection of ectopic ΔRaf22W and oncogenic Ras by immunoblot, and p110-CAAX by RT-PCR in TtH cells expressing the indicated transgenes. Total actin and GAPDH serve as loading controls.

B: Detection of phosphorylated Akt (P-Akt) and ERK1/2 (P-ERK), as a measure of PI3K and MAPK activation, in TtH cells expressing the indicated transgenes. Total AKT and ERK1/2 serve as loading controls.

C: Detection of PTEN, and as a measure of PI3K and MAPK activation, phosphorylated Akt (P-Akt) and ERK1/2 (P-ERK) in TtH cells expressing the indicated transgenes.

D: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with TtH cells expressing the following: Ras^{G12V} (white squares) or $Ras^{G12V,E37G}$ in addition to either vector (white diamonds), p110-CAAX (black squares), ΔRaf22W (black triangles), p110-CAAX + ΔRaf22W (black circles), or ΔRaf22W + PTEN (white circles).

vated effectors (Figure 3A) or Ras effector mutants (Figure 3D). Specifically, $Ras^{G12V,T35S}$ or ΔRaf22W to activate the MAPK pathway (Stanton et al., 1989; White et al., 1995), $Ras^{G12V,Y40C}$ or p110-CAAX to activate the PI3K pathway (Rodriguez-Viciana et al., 1997), or lastly, $Ras^{G12V,E37G}$ or Rlf-CAAX to activate the RalGEF pathway (White et al., 1996; Wolthuis et al., 1997) was stably expressed in ER:Ras^{G12V}-TtH cells, as assessed by immunoblot analysis (Figures 3A and 3D). In the presence of 4-OHT to activate ER:Ras^{G12V}, all six cell lines had activated MAPK, PI3K, and RalGEF pathways, as measured by an increase in ERK1/2 and AKT phosphorylation, and RalA-GTP levels, respectively (Figures 3A and 3D), and all lines grew in a soft agar, a measure of oncogenic Ras activity (Figures 3B and 3E). In the absence of 4-OHT, the targeted pathways still remained activated (Figures 3A and 3D), but consistent with previous results (Hamad et al., 2002), only cells with an activated RalGEF pathway could still grow in soft agar (Figures 3B and 3E). Thus, specific Ras effector pathways can be selectively activated in a background in which Ras expression can be controlled, providing a means to test which Ras effector pathways maintain tumor growth in the absence of oncogenic Ras.

These six cell lines and one vector control cell line were each injected into four mice given daily doses of 4-OHT to induce ER:Ras^{G12V} expression until a tumor mass was clearly visible

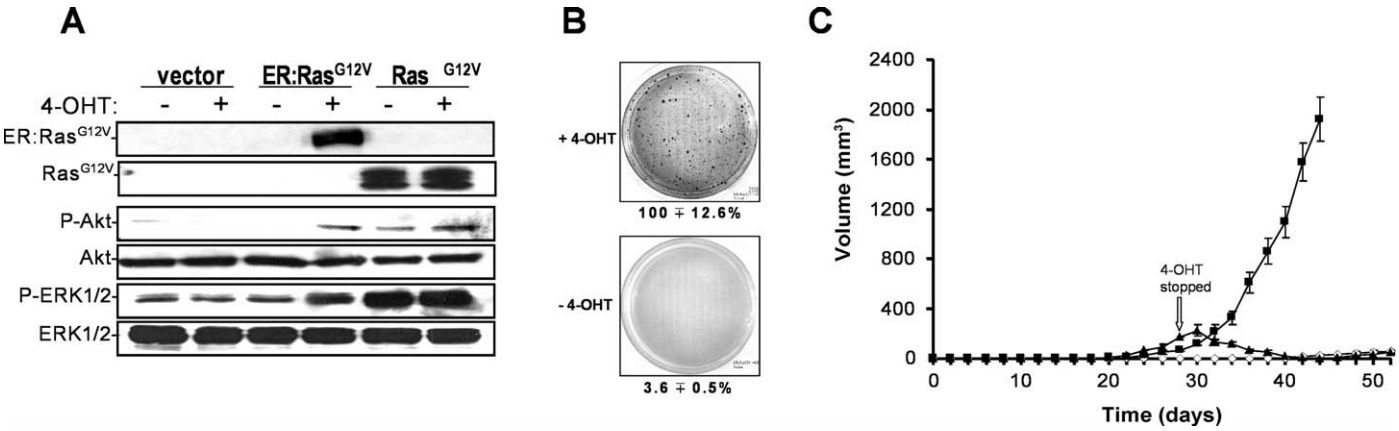

Figure 2. Oncogene addiction in human cells

A: Detection of ectopic ER:Ras^{G12V} and Ras^{G12V} by immunoblot, as well as the appropriate activation of the PI3K and MAPK pathways, as visualized by an increase in phosphorylated Akt (P-Akt) and ERK1/2 (P-ERK1/2), in TtH cells expressing the indicated transgenes in the absence (−) or presence (+) of 4-OHT. Total Akt and ERK1/2 serve as loading controls.

B: Representative image of anchorage-independent growth 3 weeks after 10^5 ER:Ras^{G12V}-TtH cells expressing no transgene (vector) were seeded in triplicate in soft agar in the absence (−) or presence (+) of 4-OHT. Below: average number of colonies and standard deviation, expressed as a percent of ER:Ras^{G12V} colony growth in the presence of 4-OHT.

C: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with TtH cells expressing vector (white diamonds) or ER:Ras^{G12V} in the presence of 4-OHT until day 28 (black triangles, arrow) or until the tumors reached maximum volume (black squares).

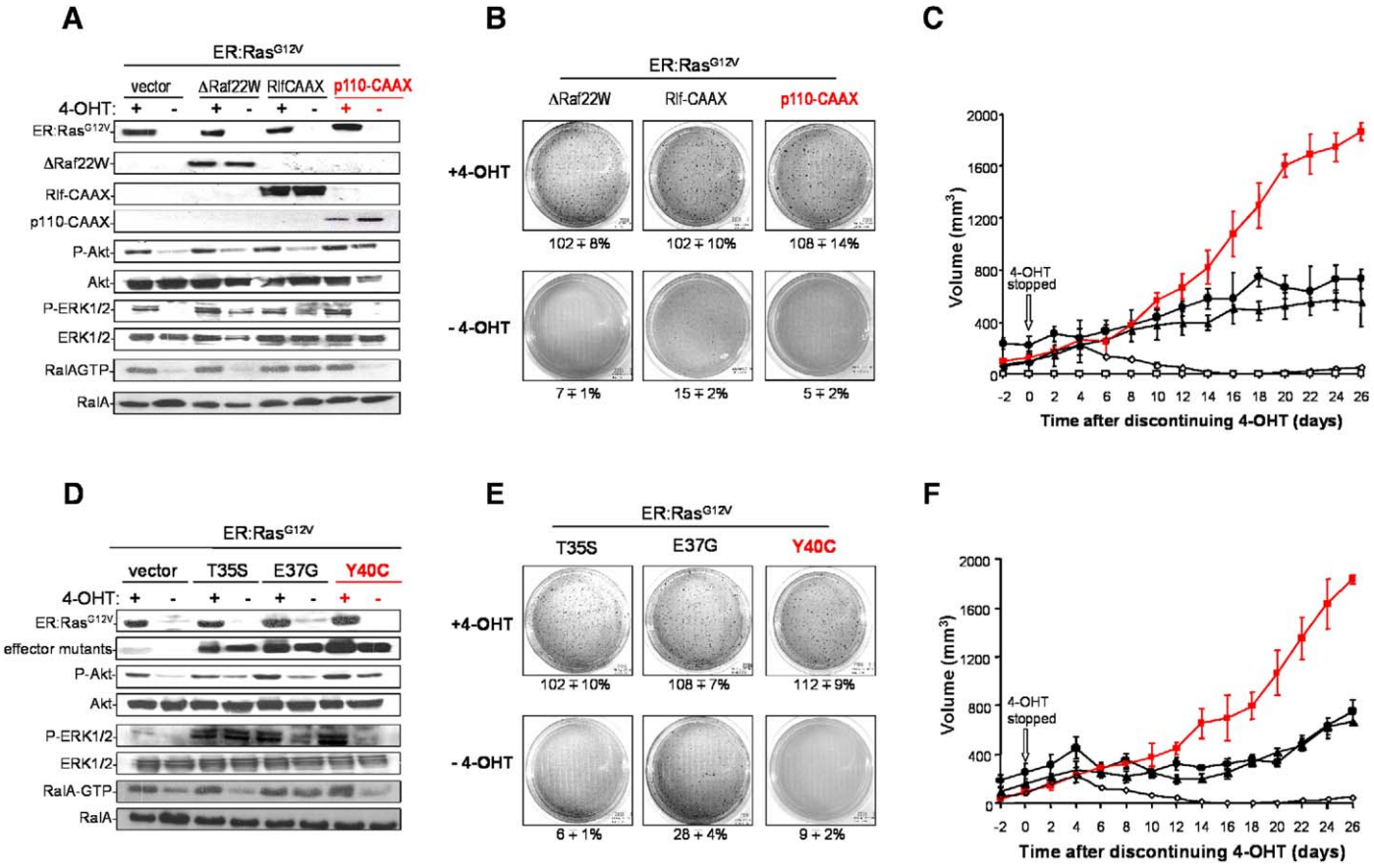

Figure 3. Reduction in the dependence upon cell-autonomous oncogenic signaling to activation of PI3K during tumor maintenance

A and D: Detection where indicated of ectopic ER:Ras^{G12V}, ΔRaf22W, Rlf-CAAX, and Ras effector mutants by immunoblot, p110-CAAX by RT-PCR, and the appropriate activation of the MAPK, PI3K, and RalA pathways, as visualized by an increase in phosphorylated Akt (P-Akt), ERK1/2 (P-ERK1/2), or the level of RalA-GTP, respectively, in ER:Ras^{G12V}-TtH cells expressing the indicated transgenes in the absence (−) or presence (+) of 4-OHT. Total Akt, ERK1/2, RalA, and GAPDH serve as loading controls. **B and E:** Representative image of anchorage-independent growth 3 weeks after 10^5 ER:Ras^{G12V}-TtH cells expressing the indicated transgene were seeded in triplicate in soft agar in the absence (−) or presence (+) of 4-OHT. Below: average number of colonies ± standard deviation, expressed as a percent of colony growth of ER:Ras^{G12V}-TtH cells infected with an empty vector. **C:** Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with ER:Ras^{G12V}-TtH cells expressing vector (white diamonds), Rlf-CAAX (black circles), p110-CAAX (red squares), or ΔRaf22W (black triangles) after 4-OHT treatments were halted (arrow) once tumors were established, or ER:Ras^{G12V}-TtH cells expressing p110-CAAX in the absence of 4-OHT throughout the entire experiment (white squares). **F:** Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with ER:Ras^{G12V} TtH cells expressing vector (white diamonds), Ras^{G12V,E37G} (black circles), Ras^{G12V,Y40C} (red squares), or Ras^{G12V,T35S} (black triangles) after 4-OHT treatments were halted (arrow) once tumors were established.

(a diameter of 0.7 cm, approximately the size of a pea). At this point, 4-OHT treatments were ceased to terminate ER:Ras^{G12V} signaling, and tumor growth was monitored for another ~25 days, or until tumors reach a maximum volume of ~1500 mm^3. Within 2 days of halting 4-OHT treatments, tumors in which either the MAPK (Ras^{G12V,T35S} or ΔRaf22W) or RalGEF (Ras^{G12V,E37G} or Rlf-CAAX) pathways were constitutively activated stopped growing and in some mice actually regressed akin to vector control tumors (Figures 3C and 3F). Conversely, tumors composed of cells expressing Ras^{G12V,Y40C} or p110-CAAX that preferentially activated PI3K, but *not* the RalGEF and MAPK pathways (Figures 3A and 3D), continued to grow in the absence of oncogenic Ras (Figures 3C and 3F). Activation of the PI3K pathway was not, however, alone sufficient to promote tumor growth, as TtH cells coexpressing ER:Ras^{G12V} and p110-CAAX injected into four mice in the absence of 4-OHT were not tumorigenic (Figure 3C). Additionally, ER:Ras^{G12V}-TtH cells expressing p110-CAAX did not acquire mutations that allowed them to grow upon suppressing ER:Ras^{G12V}. When such cells were isolated from tumors established after 4-OHT had been removed, recultured under drug selection to kill contaminating murine cells, and injected back into four mice in the absence of 4-OHT treatments, no tumors were detected (data not shown). Lastly, these results were reproducible, not only because activation of the PI3K by two different means maintained tumor growth in the absence of ER:Ras^{G12V} expression (Figures 3C and 3F), but also because expression of T-Ag, t-Ag, hTERT, ER:Ras^{G12V}, and the described activated versions of the three Ras effectors in HEK cells isolated from a completely different donor resulted in identical results, namely that only activation of the PI3K pathway could sustain tumor growth in the absence of ER:Ras^{G12V} (data not shown). We conclude that, while Ras-induced tumor initiation of TtH cells minimally requires the activation of the RalGEF, PI3K, and MAPK pathways (Figure 1D), once a tumor is formed the requirement of

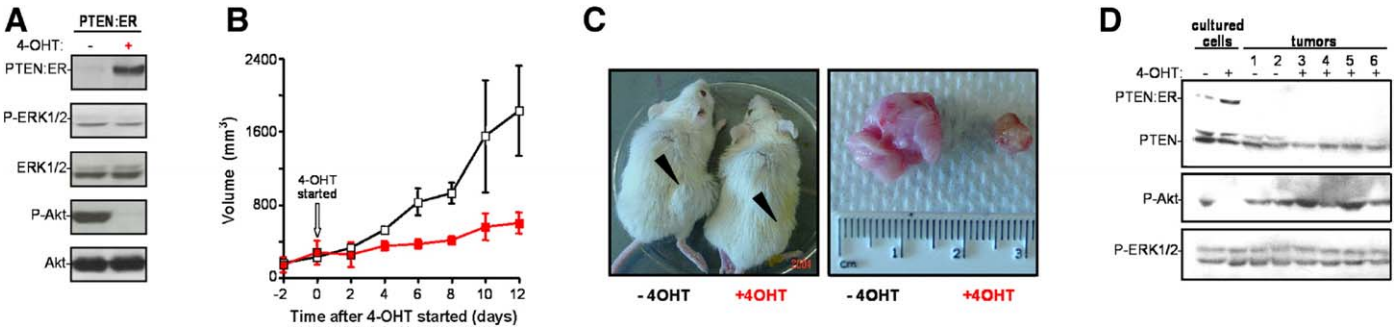

Figure 4. PI3K activation is required during tumor maintenance

A: Detection of PTEN:ER, or as a measure of activated PI3K or MAPK pathways, phosphorylated Akt (P-Akt) or ERK1/2 (P-ERK1/2), respectively, by immunoblot in the absence (−) or presence (+) of 4-OHT in Ras^{G12V}-TtH cells coexpressing PTEN:ER. Total ERK1/2 and Akt serve as loading controls.

B: Average and standard deviation of tumor volume (mm^3) from groups of ten mice versus time (days) injected with Ras^{G12V}-TtH cells expressing 4-OHT-inducible PTEN:ER either in the continued absence of 4-OHT (white squares) or upon treatments of 4-OHT to induce PTEN:ER expression once tumors reached a diameter of 0.7 cm (red squares). Day 0 (arrow): the point when tumors reached a diameter of 0.7 cm (and 4-OHT treatments were begun).

C: Representative tumors observed at the same time in mice (arrow: approximate site of injection) or when resected from one of the mice injected with Ras^{G12V}-TtH cells expressing PTEN:ER either in the absence of 4-OHT (−4OHT) or after 4-OHT treatments (+4OHT) were started at a tumor diameter of 0.7 cm. Tumors were analyzed once control (−4OHT or −dox) tumors reached maximum volume.

D: Detection of PTEN:ER and phosphorylated Akt (P-AKT) and ERK1/2 (P-ERK1/2) in Ras^{G12V}-TtH cells expressing 4-OHT-inducible PTEN:ER in culture before implanting into mice in the absence (−) or presence (+) of 4-OHT, or from cells purified from two resultant tumors grown in the absence of 4-OHT (tumors 1 and 2) or from four tumors (tumors 3–6) that eventually overcame the inhibitory effects of 4-OHT. Endogenous PTEN serves as a loading control.

Ras oncogenic signaling can be reduced to activation of the PI3K pathway (Figure 3).

Inhibition of the PI3K pathway inhibits tumor maintenance

If activation of the PI3K pathway maintained tumor growth in the absence of oncogenic Ras, we reasoned that blocking the PI3K pathway in established tumors should inhibit tumor maintenance in the presence of Ras. Akin to our approach to inducibly regulate Ras expression, we infected Ras^{G12V}-transformed TtH cells with a retrovirus encoding 4-OHT-inducible PTEN:ER, generated by fusing the mutant form of the estrogen receptor ligand binding domain to PTEN, a negative regulator of the PI3K pathway (Vivanco and Sawyers, 2002). In the absence of the 4-OHT, this fusion protein was nearly undetectable by immunoblot and unable to inhibit the PI3K pathway. Conversely, activation of PI3K by Ras^{G12V} was specifically inhibited in the presence of 4-OHT, as phosphorylation of Akt, but not ERK1/2, was lost (Figure 4A).

Having established that PI3K activity could be inducibly inhibited, we injected the resultant cells into ten mice to establish tumors, at which point six mice were treated with 4-OHT to induce PTEN:ER expression and inhibit the PI3K pathway, and as a control, four were left untreated. Tumors in the untreated mice continued to grow unabated, reaching maximum tumor volume 12 days later, whereas tumor growth was greatly inhibited in mice treated with 4-OHT (Figure 4B), resulting in visibly stunted tumor growth (Figure 4C). When tumors eventually did arise in the presence of 4-OHT, in every case (tumors 3–6, Figure 4D) there was a selection for cells that overcame 4-OHT induction of PTEN:ER expression and restored AKT activation to levels equivalent to those observed in tumors grown in the absence of 4-OHT (Figure 4D, tumors 1 and 2). Thus, continued tumor growth of established tumors was halted by inhibiting the PI3K pathway.

PI3K activation is required to maintain growth of cells in an established tumor

It is possible that, in the absence of 4-OHT, ER:Ras^{G12V} weakly activated effectors to levels that were undetectable by measuring AKT and ERK1/2 phosphorylation and RalA-GTP levels, which in turn cooperated with p110-CAAX to foster tumor growth once 4-OHT treatments were halted. To definitively test if autocrine activation of the PI3K pathway is indeed *alone* sufficient to support tumor growth in the absence of Ras, and does not rely on possible weak activation of other Ras effectors by low levels of ER:Ras^{G12V}, we tested whether nonmalignant TtH cells expressing p110-CAAX (but *not* ER:Ras^{G12V}) were tumorigenic once tumor growth was initiated by completely different Ras^{G12V}-expressing TtH cells (Figure 5A). Four mice were injected with a mixture of TtH cells stably expressing either Ras^{G12V} (to establish a tumor) or p110-CAAX (and the visual marker LacZ to identify these cells). As a control, we similarly injected four mice each with a mixture of TtH cells expressing Ras^{G12V} and TtH cells coexpressing LacZ and either an empty vector, ΔRaf22W, to activate the MAPK pathway, or Rlf-CAAX to activate the RalGEF pathway.

The mixture of Ras^{G12V} cells and p110-CAAX cells led to tumors that grew faster than any of the other combinations tested (Figure 5C). Moreover, when two tumors were excised and treated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to stain LacZ-expressing p110-CAAX cells blue, the tumors exhibited prominent blue staining (Figure 5B). To ascertain more accurately the contribution of p110-CAAX cells in the tumor, the two remaining tumors were resected, and individual cultures were established in the presence of drug selection to kill contaminating murine cells and enrich for the injected human cells. Upon treatment of the resultant cultures with X-gal, almost one-quarter of the cells stained strongly for LacZ, with many more being weakly positive (Figure 5B). This number far exceeds the amount injected, indicat-

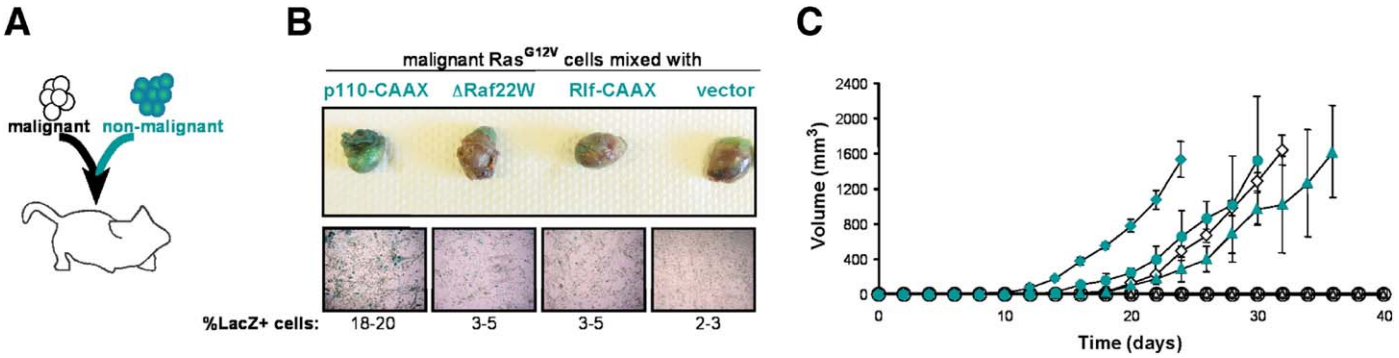

Figure 5. Activation of PI3K maintains tumor growth in established tumors

A: Diagram of mixing experiment involving Ras^{G12V}-TtH cells (malignant) to establish a tumor with LacZ-positive TtH cells expressing different activated versions of Ras effectors (nonmalignant).

B: Four mice were injected with a mixture of Ras^{G12V}-TtH cells and LacZ-positive TtH cells expressing the indicated transgenes, after which tumors were resected when they reached maximum volume, and two were immediately treated with X-gal (representative tumor, top panel) and two were adapted to grow in culture and then treated with X-gal to visualize LacZ-positive cells (representative culture, bottom panel).

C: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with a mixture of vector and LacZ-positive TtH cells (white squares) or a mixture of Ras^{G12V}-TtH cells and LacZ-positive TtH cells expressing no transgene (vector, white diamonds), ΔRaf22W (blue triangles), Rlf-CAAX (blue circles), or p110-CAAX (blue diamonds) or injected with LacZ-positive TtH cells expressing p110-CAAX that were purified from two different tumors established when these cells were originally mixed with Ras^{G12V}-TtH cells (white circles and triangles).

ing that the p110-CAAX TtH cells proliferated in what could best be described as a parasitic fashion, greatly contributing to the malignant mass of the tumors while on their own remaining unable to initiate tumor growth.

The p110-CAAX cells in the tumors did not acquire any additional changes to allow them to grow in this malignant fashion. Neither the original p110-CAAX/LacZ cells nor those isolated from two different tumors (with Ras-transformed cells removed by drug selection) and reinjected into four mice each formed tumors (Figure 5C). Similarly, neither vector control nor those cells with a constitutively active MAPK or RalGEF pathway contributed significantly to the tumors (Figure 5B). Thus, in the complete absence of oncogenic Ras, activation of the PI3K pathway, and not other Ras effectors, sustains tumor growth.

Ras effector activation in established tumors

The reduced requirement for Ras oncogenic signaling during tumor maintenance could be due either to a frank lack of any need for MAPK and RalGEF activity, or to Ras-independent stimulation of these pathways once a tumor is established. To address these two possibilities, we first assayed whether tumors maintained by p110-CAAX had activated the MAPK and RalGEF pathways in vivo, despite lacking ER:Ras^{G12V} expression and not activating these pathways in culture. Three different tumors derived from ER:Ras^{G12V} TtH cells expressing p110-CAAX were resected from mice ~25 days after 4-OHT treatments were halted, well beyond the point when ER:Ras^{G12V} expression was extinguished in culture after 4-OHT removal, and assayed for ER:Ras^{G12V} expression and activation of PI3K, MAPK, and RalGEF pathways. As expected, ER:Ras^{G12V} was not detected in these tumors, as they were grown in the absence of 4-OHT, and the PI3K pathway was activated (Akt was phosphorylated), owing to ectopic p110-CAAX expression. However, even though ER:Ras^{G12V} was not expressed in these tumors, and these cells did not activate the MAPK or RalGEF pathways in the absence of 4-OHT in culture, in the tumors ERK1 and ERK2 were phosphorylated, a measure of MAPK activation, and the levels of RalA-GTP were elevated, a measure of RalGEF activation (Figure 6A). Thus, the remaining Ras effector pathways were activated in tumors maintained by PI3K activity in the absence of oncogenic Ras expression.

We next addressed whether this activation of MAPK and RalGEF was important for tumor maintenance in the absence of oncogenic Ras expression. In the case of the MAPK pathway, Ras^{G12V}-transformed TtH cells were infected with a retrovirus encoding 4-OHT-inducible MEK^{DN}:ER that we generated by fusing the mutant form of the estrogen receptor ligand binding domain to the K_{97}-M dominant-negative (DN) version of MEK1, which is well established to inhibit the MAPK pathway (Mansour et al., 1994). In the absence of 4-OHT, this fusion protein was modestly expressed, but not at a level to robustly inhibit the MAPK pathway. In the presence of 4-OHT, the protein was abundantly expressed and clearly able to specifically inhibit activation of MAPK pathway by Ras^{G12V}, as ERK1/2 phosphorylation was abolished without affecting the level of AKT phosphorylation (Figure 6B). The resultant cells were injected into eight mice to establish tumors, after which half the mice were treated with 4-OHT to induce MEK^{DN}:ER expression and inhibit the MAPK pathway, and as a control, half were left untreated. Tumors in untreated mice continued to grow unabated, reaching maximum tumor volume 10 days later, whereas tumor growth was inhibited in mice treated with 4-OHT (Figure 6E), resulting in visibly stunted tumor growth (Figure 6D).

In the case of the RalGEF pathway, *RalA* shRNA has been shown to inhibit RalGEF-induced transformation, and in a specific manner, as this effect was rescued by expression of a siRNA-resistant RalA protein (Lim et al., 2005). To generate an inducible *RalA* shRNA, Ras^{G12V}-transformed TtH cells were engineered to express the TET repressor protein, which can be inhibited by the molecule dox. The cells were then infected with a retrovirus encoding *RalA* shRNA downstream of the *TET*

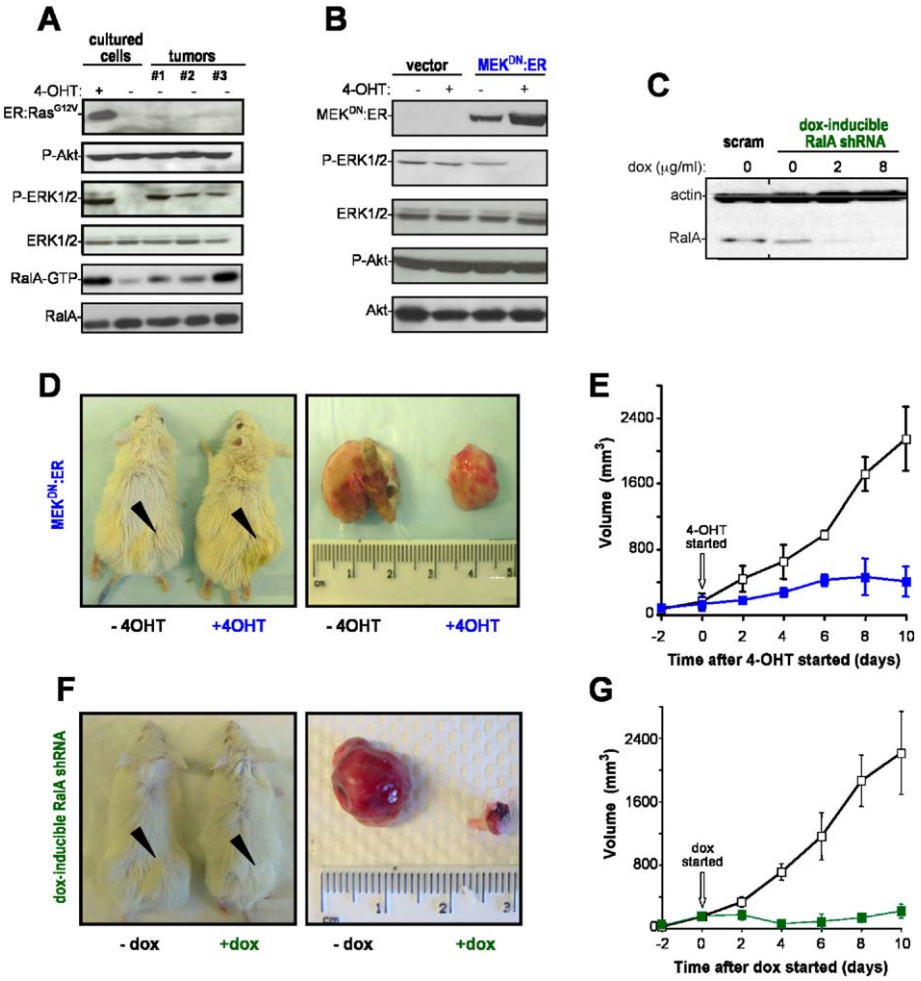

Figure 6. A role of MAPK and RalGEF pathways in tumor maintenance

A: Cultured ER:Ras^{G12V}-TtH cells expressing p110-CAAX in the absence (−) or presence (+) of 4-OHT or the resultant tumors derived from these cells once maximum tumor volumes were reached, after 4-OHT treatments were halted, were immunoblotted to detect ER:Ras^{G12V}, phosphorylated AKT (P-Akt), or ERK1/2 (P-ERK1/2) and RalA-GTP as measures of activation of the PI3K, MAPK, and RalGEF pathways, respectively.

B: Detection of MEK^{DN}:ER or phosphorylated AKT (P-Akt) or ERK1/2 (P-ERK1/2) as measures of activation of the PI3K or MAPK pathways, respectively, by immunoblot in Ras^{G12V}-TtH cells expressing 4-OHT-inducible MEK^{DN}:ER to inhibit the MAPK pathway or a vector control in the absence (−) or presence (+) of 4-OHT. Total ERK1/2 and Akt serve as loading controls.

C: Detection of endogenous levels of RalA by immunoblot in Ras^{G12V}-TtH cells stably expressing dox-inducible RalA shRNA to inhibit the RalGEF pathway or a scramble control in the presence of increasing concentrations of dox.

D and E: Representative tumors in mice (arrow: approximate site of injection) or when resected from one of four mice injected with Ras^{G12V}-TtH cells containing 4-OHT-inducible MEK^{DN}:ER either in the absence of the activating agent (−4OHT, white squares) or after 4-OHT treatments were started (+4OHT, blue squares) at a tumor diameter of 0.7 cm. All tumors were analyzed once control (−4OHT) tumors reached maximum volume.

F and G: Average and standard deviation of tumor volume (mm^3) from groups of eight mice versus time (days) injected with Ras^{G12V}-TtH cells containing dox-inducible RalA shRNA either in the continued absence of the activating agent (−dox, white squares) or upon treatments with dox once tumors reached a diameter of 0.7 cm (+dox, green squares). Day 0 (arrow): the point when tumors reached a diameter of 0.7 cm and 4-OHT or dox treatments were initiated to inhibit the MAPK or RalGEF pathway, respectively.

operator and, when exposed to dox, were shown to have a dose-dependant decrease in endogenous RalA protein levels, as assessed by immunoblot (Figure 6C). These cells were then injected into eight mice, allowed to form tumors, and then either treated with dox to induce *RalA* shRNA or left untreated. In this case, inhibition of the RalGEF pathway actually caused tumor regression in some mice (Figure 6G), and resected tumors were substantially reduced in their mass (Figure 6F). Thus, activation of the MAPK and RalGEF pathways is still required for tumor maintenance, although this activation is independent of oncogenic Ras.

The PI3K/AKT pathway prevents apoptosis during tumor maintenance

While the MAPK and RalGEF pathways can be activated in the absence of oncogenic Ras once a tumor is established, PI3K was the only effector that still had to be activated in an autonomous fashion in tumor cells. We therefore addressed what function of oncogenic Ras was so critical that it could not be lost during tumor maintenance. As a starting point, we compared the effects of the loss of oncogenic Ras in the presence and absence of p110-CAAX on tumor histology. Twelve mice were injected with ER:Ras^{G12V}-TtH cells expressing either p110-CAAX or no transgene (vector). Tumors were allowed to reach a diameter of 0.7 cm in the presence of 4-OHT, after which these treatments were ceased, and 3, 5, 9, 12, 15, and 20 days later one mouse from each of the two treatment groups was photographed or a tumor was removed and analyzed for apoptosis (TUNEL-positive), cell proliferation (Ki67-positive), and/or general histology (H&E staining). In vector control cells, there was a clear increase in TUNEL-positive (Figure 7A) cells and a decrease in Ki67-positive (Figure 7B) cells at day 5, followed by a dramatic collapse of tumor cellular architecture and appearance of hemorrhagic foci, nuclear and cytoplasmic debris at day 9 (Figure 7C), the near complete loss of overall tumor structure at day 15 (Figure 7D), and the absence of a visible tumor mass at day 20 (Figure 7E). During the same time course, p110-CAAX-expressing tumors continued to grow and did not show these profound changes in tumor histology (Figures 7A–7E).

As cellular apoptosis preceded the demise of vector control tumors upon silencing ER:Ras^{G12V} expression, we speculated

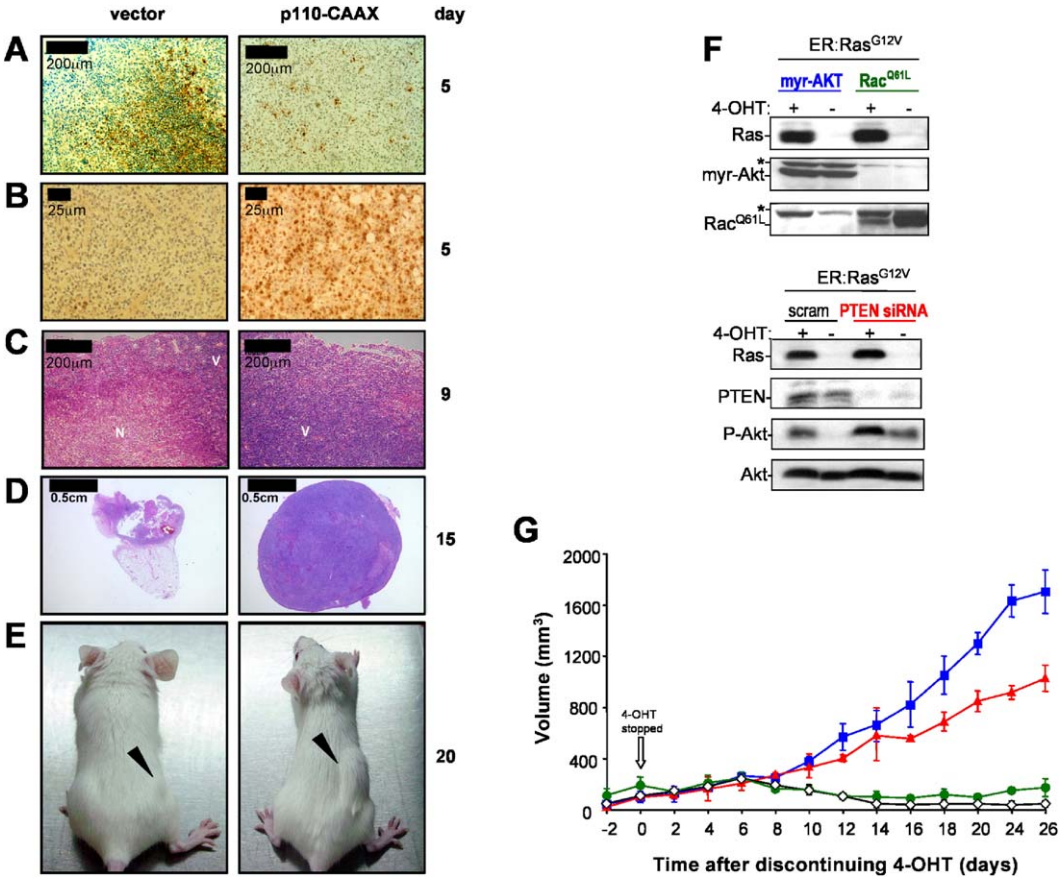

Figure 7. The Akt pathway prevents apoptosis during tumor maintenance

A–E: Mice were injected with ER:Ras^{G12V}-TtH cells expressing vector or p110-CAAX, tumors were established, then 4-OHT treatments were halted; at the indicated times afterwards one tumor from each treatment group was sectioned and H&E stained and assayed for TUNEL-positive (dark brown) cells (**A**); assayed for Ki67-positive (dark brown) cells (**B**); or H&E-stained (V, viable regions; N, necrotic regions) (**C and D**). **E:** Mice harboring tumors derived from ER:Ras^{G12V}-TtH cells expressing vector or p110-CAAX were photographed 20 days after 4-OHT treatments were halted (arrow: approximate site of injection).

F: Detection of ectopic myr-Akt, Rac^{Q61L}, ER:Ras^{G12V}, and endogenous PTEN by immunoblot, or the appropriate activation of the PI3K pathway, as visualized by an increase in phosphorylated Akt (P-Akt), in TtH cells expressing ER:Ras^{G12V} and myr-Akt, Rac^{Q61L}, a vector encoding a siRNA specific for PTEN (PTEN siRNA), or the corresponding scrambled sequence (scram) in the presence (+) or absence (−) of 4-OHT. Akt serves as a loading control. *Nonspecific band.

G: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with ER:Ras^{G12V}-TtH cells expressing myr-Akt (blue squares), Rac^{Q61L} (green circles), PTEN siRNA (red triangles), or scrambled control (white diamonds) after 4-OHT treatments were halted (arrow).

that the effects of p110-CAAX might be related to promoting cell survival. In agreement with this hypothesis, we show that of two well-established downstream targets of PI3K, the pro-growth and survival protein AKT (Vivanco and Sawyers, 2002), but not Rac, a GTPase that affects the actin cytoskeleton (Burridge and Wennerberg, 2004), maintained tumor growth in the absence of Ras. Specifically, a constitutively active version of AKT (myr-AKT [Kulik et al., 1997]) or Rac (Rac^{Q61L} [Singh et al., 2004]) was stably expressed in the ER:Ras^{G12V}-TtH cells (Figure 7F), and the resultant cell lines were injected into four mice each treated daily with 4-OHT until tumors were established, after which time these treatments were stopped to terminate the ER:Ras^{G12V} signaling. Cells expressing myr-AKT continued to grow in the absence of oncogenic Ras, whereas those expressing Rac^{Q61L} regressed (Figure 7G).

The PI3K pathway is negatively regulated by the tumor suppressor PTEN, and PTEN expression is lost in many types of cancer cells, leading to AKT activation (Vivanco and Sawyers, 2002). Thus, as a means to activate the PI3K pathway in a more physiological way, we tested whether downregulation of PTEN could maintain tumor growth in the absence of Ras^{G12V}. ER:Ras^{G12V}-TtH cells were stably infected with a retrovirus encoding a *PTEN* shRNA, which led to a decrease in PTEN protein levels and activation of AKT in the absence of ER:Ras^{G12V} (Figure 7F). The resulting population injected into four mice readily formed tumors in mice that continued to grow unabated when oncogenic Ras signaling was silenced by halting 4-OHT treatments (Figure 7G). Conversely, tumors derived from control cells expressing a scrambled version of the shRNA that failed to inhibit *PTEN* expression (Figure 7F) regressed upon halting 4-OHT treatments (Figure 7G). These effects were limited to tumor maintenance, as ER:Ras^{G12V}-TtH cells expressing either *PTEN* shRNA or myr-AKT were not tumorigenic in the absence of 4-OHT (data not shown). We surmise from these

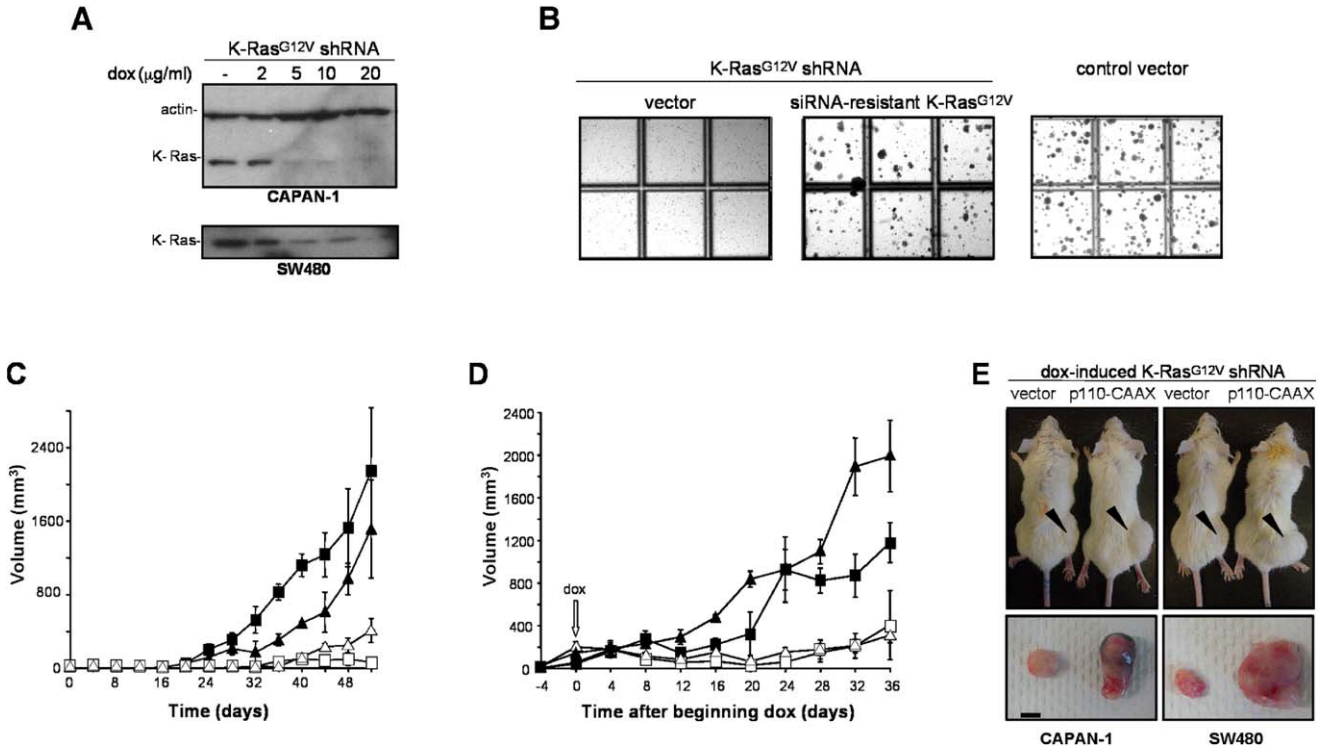

Figure 8. Activation of PI3K maintains tumor growth in the absence of oncogenic Ras in human cancer cell lines

A: Detection of endogenous K-Ras knockdown by immunoblot in the human CAPAN-1 (pancreatic) and SW480 (colon) cancer cell lines harboring dox-inducible shRNA specific for the endogenous oncogenic (G12V) *K-Ras* allele in the presence of increasing levels of dox. Total actin serves as a loading control.

B: Rescue of *K-Ras*^{G12V} shRNA. Representative image of anchorage-independent growth 3 weeks after 10^5 CAPAN-1 cells expressing control vector or *K-Ras*^{G12V} shRNA (noninducible) and either a control vector or one encoding siRNA-resistant K-Ras^{G12V} were seeded in triplicate in soft agar.

C: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with SW480 or CAPAN-1 cells constitutively expressing both *K-Ras*^{G12V} shRNA and siRNA-resistant K-Ras^{G12V} (black squares or black triangles, respectively), or SW480 or CAPAN-1 cells expressing dox-inducible *K-Ras*^{G12V} shRNA and p110-CAAX in the continued presence of dox (white squares or white triangles, respectively).

D: Average and standard deviation of tumor volume (mm^3) from groups of four mice versus time (days) injected with CAPAN-1 cells expressing dox-inducible *K-Ras*^{G12V} shRNA and either a control vector (white triangles) or one encoding p110-CAAX (black triangles) or SW480 cells expressing dox-inducible *K-Ras*^{G12V} shRNA and either a control vector (white squares) or one encoding p110-CAAX (black squares) after dox treatments were initiated (arrow).

E: Representative tumors in mice (top, arrow, location of injection) and when resected (bottom) after the described cells were injected in mice treated with dox once tumors were established to inhibit the expression of the *K-Ras*^{G12V} allele. Tumors were viewed 36 days after dox treatments were begun.

gain- and loss-of-function experiments that, once tumor growth has been initiated, one of the main functions of oncogenic Ras is to protect neoplastic cells from an apoptotic fate by activation of the AKT pathway.

Activation of PI3K is required to maintain growth of human cancer cell lines in the absence of oncogenic Ras

While we demonstrate using multiple assays that genetically engineered human tumor cells depend upon PI3K activation for tumor maintenance, we wished to address if the same holds true in actual cancer cell lines isolated from patients diagnosed with cancer in which *Ras* mutations are present. Moreover, our studies were performed with *H-Ras*, whereas most tumors have *K-Ras* mutations (Bos, 1989), and H- and K-Ras can activate PI3K pathway to different extents (Yan et al., 1998). We therefore introduced a dox-inducible shRNA specific for the endogenous oncogenic G12V allele of *K-Ras* (and the TET repressor protein) into CAPAN-1 and SW480 cells, two cell lines derived from completely different human cancers that are known to express this mutant allele (Brummelkamp et al., 2002; Ikehara et al., 2005), and showed by immunoblot that addition of dox to the resultant cell lines led to a dose-dependant decrease in endogenous K-Ras protein levels (Figure 8A). The ability of this shRNA sequence to suppress oncogenic *Ras* expression was reflected in assays of Ras oncogenesis, as CAPAN-1 cells engineered to constitutively express the described *K-Ras*^{G12V} shRNA (and an empty vector) failed to grow in soft agar. Coexpression of a siRNA-resistant K-Ras^{G12V} protein in these cells restored soft agar growth to the level of vector control cells lacking any shRNA sequence (Figure 8B), indicating that the effects of the shRNA were a consequence of knocking down *K-Ras*^{G12V} expression. Similar observations were found in assays of tumor growth. Specifically, tumor initiation of CAPAN-1 and SW480 cells expressing dox-inducible shRNA (and p110-CAAX; see below) in the presence of constant dox treatments was greatly retarded. Again, this reduc-

tion in tumor growth was rescued in both cell lines in which the constitutive knockdown of *K-Ras*^{G12V} was complemented by expression of a siRNA-resistant K-Ras^{G12V} protein (Figure 8C). Thus, we demonstrate that endogenous *K-Ras*^{G12V} can be specifically knocked down in two human cancer cell lines, resulting in a reduction of Ras-mediated transformation and tumorigenesis.

To next test if activation of the PI3K pathway could rescue the loss of Ras function during tumor maintenance, p110-CAAX or a control vector was introduced into the two cell lines engineered to express dox-inducible *K-Ras*^{G12V} shRNA. The resultant four populations were then each injected into four mice in the absence of dox to allow *K-Ras*^{G12V} expression and establish tumors. Upon initiation of dox treatments to knock down *K-Ras*^{G12V} expression, vector control cells began to regress, whereas those tumors expressing p110-CAAX continued to grow (Figure 8D), resulting in a clearly visible difference in the tumor size between vector- and p110-CAAX-expressing cells (Figure 8E). Activation of the PI3K pathway was not, however, alone sufficient to promote tumor growth in the absence of Ras^{G12V}. Tumor initiation was greatly reduced or abolished in the two cell lines harboring dox-inducible *K-Ras*^{G12V} shRNA and p110-CAAX when injected into mice in the presence of dox (to constitutively inhibit K-Ras^{G12V} expression), and in the cases where tumors masses were detected, the tumors grew extremely poorly (Figure 8C). Thus, activation of the PI3K pathway maintained the tumorigenic growth of not only engineered tumor cells in the absence of Ras, but also human cancer cell lines.

Reduction in the requirements of oncogenic Ras during tumorigenesis

Tumorigenesis is a dynamic process, and hence we addressed whether Ras must activate the same signaling pathways during tumor initiation as during tumor maintenance. We now demonstrate that, while multiple arms of oncogenic Ras signaling are required for tumor initiation, oncogenic activation of the downstream PI3K/AKT pathway is sufficient for tumor maintenance, as (1) PI3K or AKT activation maintained tumor growth upon loss of ER:Ras^{G12V} expression even though these molecules did not activate other Ras effectors; (2) inducible expression of PTEN stopped continued tumor growth of established tumors; and (3) PI3K activation in otherwise nonmalignant cells permitted the cells to grow in a malignant fashion in established tumors. Thus, while cancers may become "addicted" to the initiating oncogene (Giuriato et al., 2004), our experiments suggest that the full might of oncogenes is much more important to initiate tumors, and that only residual activities are essential, at least in the case of *Ras*, to maintain tumor growth.

Why cancer cells can lose their dependency on oncogenic Ras still remains to be resolved, but we speculate that, once a tumor microenvironment is established, tumor vasculature or stromal cells supply paracrine factors that activate Ras effector pathways (Mueller and Fusenig, 2004). Additionally, we demonstrate that, in the absence of oncogenic Ras, the MAPK and RalGEF pathways become activated in tumor cells independently of p110-CAAX expression once a tumor is established, and that the cells depended upon these pathways for continued tumor growth. In fact, an established tumor (microenvironment) even fostered the growth of nonmalignant cells, provided the crucial PI3K/AKT pathway was activated. Perhaps, then, similar premalignant cells generated during tumor evolution in human cancers capitalize on the tumor microenvironment and similarly grow in a parasitic fashion. Hence, a reduction in the dependency for oncogenic signals as tumors progress could lead to the expansion of cells with different levels of malignancy, possibly accounting for some of the known heterogeneity of the malignant fraction of solid tumors.

Lastly, the discovery that PI3K activation was critical during tumor maintenance highlights the importance of this pathway as an anticancer target. We further distilled this pathway to activation of AKT, consistent with the observation that this pathway is commonly activated in human cancers. Importantly, these observations were extended to human cancer cell lines, suggesting that the importance of this pathway in experimental systems of tumor maintenance may apply to actual human cancers. Since cancers are treated at the tumor maintenance stage, targeting this aspect of Ras oncogenesis may hold promise as an approach to the management of Ras-driven human cancers.

Experimental procedures

Cell lines and plasmids

Primary HEK cells (kind gifts of Silvia Bacchetti) stably expressing the early region of SV40 (encoding large T-Ag and small t-Ag) and the hTERT catalytic subunit of telomerase, CAPAN-1 cells (Brummelkamp et al., 2002), or SW480 cells (Ikehara et al., 2005) were stably infected to generate polyclonal populations as previously described (Hamad et al., 2002) with the indicated retrovirus(es) derived from pBabe, pBabePuro, pBabeZeo, or pBabeBlast with no insert or by subcloning into these vectors cDNAs encoding H-Ras^{G12V}, H-Ras^{G12V,E37G}, H-Ras^{G12V,Y40C}, H-Ras^{G12V,T35S}, p110-CAAX, Rlf-CAAX, ΔRaf22W (Hamad et al., 2002), ER:H-Ras^{G12V} (a kind gift of Paul Khavari), myr-AKT-HA (Kulik et al., 1997), HA-Rac^{Q61L} (a kind gift from Channing Der), PTEN (subcloned without HA tag from pCDNA3-PTEN-HA; a kind gift of Chris Kontos), FLAG-MEK^{DN}:ER (the MEK1 cDNA derived from pEN-MEK1 [ATCC] was mutated by site-directed mutagenesis from K_{97} to M [Mansour et al., 1994] and cloned in frame with a N-terminal FLAG epitope tag by PCR upstream and in frame with the ER domain derived from the aforementioned ER:H-Ras^{G12V} cDNA), PTEN:ER (generated by cloning the aforementioned PTEN cDNA in frame upstream of the aforementioned ER sequence), LacZ (subcloned from pCMV-β-Gal [Clontech]), or siRNA-resistant K-Ras^{G12V} (in which the K-Ras^{G12V} cDNA, a kind gift of Channing Der, was engineered by site-directed mutagenesis to contain the silent mutation changes T_{36}GGC to AGGA). For shRNA experiments, the same cell lines were stably infected with the indicated retroviruses derived from pSUPER-RETRO-PURO (Oligoengine) containing the shRNA *PTEN* sequence CCCAGTCAGAGGCGCTATGTG or the scrambled derivative CCAAGCCAGAGACGTTACGTA, or pSUPER-RETRO-GFP/NEO containing *K-Ras*^{G12V}-specific shRNA (Brummelkamp et al., 2002). To achieve inducible shRNA expression, cells were coinfected with pCMVneo (Bender et al., 1987) encoding the TET repressor derived from pcDNA6-TR (Invitrogen) and pSUPER-RETRO-PURO-TETO, engineered from pSUPER-RETRO-PURO (Brummelkamp et al., 2002) to contain the *TET* operator (downstream of the *H1* promoter) and either no insert, shRNA specific for the G12V version of *K-Ras* (Brummelkamp et al., 2002), or RalA shRNA (Lim et al., 2005).

Tumors and cell culture

SCID/beige mice were given seven daily intraperitoneal injections of 1 mg 4-OHT dissolved in 0.1 ml of peanut oil (Sigma) or, when indicated, peanut oil alone, akin to methods previously described (Pelengaris et al., 2002), then injected subcutaneously in the flanks with 10^7 TtH cells expressing 4-OHT ER:Ras^{G12V}, followed by daily injections of 4-OHT until (unless otherwise specified) tumors reached a diameter of 0.7 cm. In some cases, 4-OHT treatments were supplemented by implanting subcutaneously a pellet containing 10 mg of 4-OHT. In the case of CAPAN-1 and SW480 cells containing dox-inducible *K-Ras*^{G12V} shRNA or Ras^{G12V}-TtH cells containing dox-inducible *RalA* shRNA, or 4-OHT-inducible MEK^{DN}:ER or PTEN:ER,

mice were supplied with water containing 1 mg/ml of dox hydrochloride (Sigma) or injected with 4-OHT as above after tumors reached a diameter of 0.7 cm (unless otherwise specified). Four or more mice were used per treatment group in all experiments except when noted.

H&E staining, Ki67 immunohistochemistry (α-Ki67 antibody; Vector Laboratories), and the TUNEL assay (Apoptag+ Peroxidase in situ Detection Kit; Chemicon International) were performed on the indicated tumor sections using standard assays. For mixing experiments, 9×10^6 Ras^{G12V}-TtH cells were mixed with 9×10^6 TtH cells expressing LacZ and p110-CAAX, ΔRaf22W, Rlf-CAAX, or no transgene (vector) and similarly injected into the flanks of four mice. Once tumors were established, two tumors were treated with X-gal using standard protocols to identify LacZ-positive cells. The two other tumors were established in culture under G418 selection, the resulting cell culture was stained with X-gal, and strongly positive blue cells were counted.

In cases where tumor-derived cells were retested for tumor growth, tumors were established in culture under puromycin selection to enrich for TtH cells, except in the case of mixing experiments, in which cells were selected under G418. The latter cells were also subjected to another round of puromycin selection to specifically select LacZ + p110-CAAX TtH cells. Cells were injected and assayed for tumor growth as described above. Experimental procedures involving mice were reviewed and approved by the Duke University Institutional Animal Care and Use Committee.

Molecular analysis

The described Ras proteins, ΔRaf22W, myr-AKT-HA or HA-Rlf-CAAX or HA-Rac^{Q61L}, PTEN or PTEN:ER, actin, endogenous and ectopic K-Ras, FLAG-MEK^{DN}:ER, and RalA were detected by standard immunoblot analysis using the primary antibodies α-Pan-Ras (Oncogene), α-Raf-1 (Santa Cruz), α-HA (Roche), α-PTEN (Cell Signaling Technology), α-actin (Santa Cruz), α-K-Ras (Santa Cruz), α-FLAG (Sigma), and α-RalA (Transduction Laboratories), respectively. p110-CAAX mRNA was detected by reverse transcription PCR amplification, as previously described (Hamad et al., 2002). MAPK, PI3K, and RalA pathway activation was determined by culturing cells in 0.5% fetal bovine serum for 48 hr in the presence or absence of 1 μM 4-OHT (Sigma). Derived cell lysates were immunoblotted as previously described (Hamad et al., 2002) to detect AKT or the activated S473 phosphorylated form, or ERK1/2 or the activated Thr202/Tyr204 phosphorylated forms, using the primary antibodies α-Akt, α-phospho(Ser 473)-Akt (Cell Signaling Technology), K-23 α-ERK1/2 (Santa Cruz), and E10 α-phospho(Thr202/Tyr204)-p42/44 MAPK (Cell Signaling Technology), respectively, or to detect Ral-GTP, RalA captured with bacterially expressed GST-RalBD of RalBP1 followed by immunoblot analysis with the aforementioned RalA antibody, respectively, as previously described (Wolthuis et al., 1998).

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